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AN ODOROUS COMPOUND IN SKIM MILK CULTURES OF ACHROMOBACTER PUTREFACIENS

(A Common Causal Agent for Surface Taint Butter)

W. L. Dunkley

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AN ODOROUS COMPOUND IN SKIM MILK CULTURES OF ACHROMOBACTER PUTREFACIENS (A COMMON CAUSAL AGENT FOR SURFACE TAINT BUTTER)

by

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the requirements for the degree of
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CONTENTS

INTRODUCTION 1

METHODS 2

PART 1 - PRELIMINARY INVESTIGATION TO DETERMINE CHARACTERISTIC PROPERTIES OF THE ODOROUS COMPOUND

INTRODUCTION 5

EXPERIMENTAL 5

SUMMARY 8

PART 2 - SEPARATION AND IDENTIFICATION OF VOLATILE ACIDS

INTRODUCTION 9

REVIEW OF THE LITERATURE 9

EXPERIMENTAL 14

SUMMARY 27

PART 3 - QUANTITATIVE DETERMINATIONS OF VOLATILE ACIDS IN STEAM DISTILLATE RESIDUES

INTRODUCTION 30

EXPERIMENTAL 30

SUMMARY 34

DISCUSSION 35

SUMMARY AND CONCLUSIONS 52

APPENDIX - THE DUCLAUX DISTILLATION METHOD

REVIEW OF THE LITERATURE 55

METHOD 57

EXPERIMENTAL 63

DISCUSSION 65

SUMMARY AND CONCLUSION 69

PLATES 71

TABLES 73

REFERENCES 95

AN ODOROUS COMPOUND IN SKIM MILK CULTURES OF ACHROMOBACTER PUTREFACIENS (A COMMON CAUSAL AGENT FOR SURFACE TAINT BUTTER)

INTRODUCTION

"Surface taint" is a butter defect which is characterized by an objectionable odor and flavor. In spite of the fact that the odor of surface taint butter is rather specific and definitely characteristic, and that the defect has been extensively studied, the compound responsible for the odor associated with the defect and the source of this compound in milk have not yet been determined. The work reported in this thesis was undertaken jointly by the Departments of Biochemistry and of Dairying, University of Alberta, and Division of Bacteriology and Dairy Research, Science Service, Ottawa, to investigate the substance responsible for the characteristic odor of surface taint butter.

The defect called surface taint has been adequately described and its history thoroughly reviewed by Wolochow (1940). Linneboe (1940) reported statistics showing the incidence of surface taint in Alberta for the years 1926 to 1939 inclusive. A brief discussion of the defect will therefore suffice here.

The name "surface taint" is applied to a defect of butter characterized by an unpleasant odor suggesting putrefaction which is usually detected from surfaces of affected butter only after exposure to the air for some time. Numerous terms have been used to describe surface taint butter, among them "peculiar sweetish flavor somewhat resembling that of condensed milk", "gutted hog", "turnipy", "sickening", "the limburger defect", "putrid type of cheesiness", "rotten cabbage", and "sweaty feet". To those associated with the present work, "sweaty feet" seemed to give the closest approximation.

It has been commonly agreed that the organism _Achromobacter putrefaciens_ causes surface taint when it is inoculated into cream or butter wash water before churning. (Derby and Hammer, 1931; Cullity and Griffin, 1938; and Wolochow, 1940).

METHODS

Ach. putrefaciens, when grown in skim milk, produced an odor suggestive of sweaty feet which resembled the odor of surface taint butter. It seemed that the compound responsible for the odor of skim milk cultures of _Ach. putrefaciens_ might be the same as that giving the characteristic odor to surface

taint butter. (Wolochow, 1940). Hence, in this investigation, skim milk cultures of *Ach. putrefaciens* were used as the source of the odorous compound which was to be identified. But, as the identity of the odors cannot be taken for granted, it is thought best at this stage to retain the distinction between the odor of skim milk cultures of *Ach. putrefaciens* and the typical odor of surface taint butter. For this reason, the odor produced in skim milk by *Ach. putrefaciens* has been called the sweaty feet odor, and that produced in butter, the surface taint odor.

The strain of *Ach. putrefaciens* used in this study was isolated and identified by Dr. J. B. Linneboe, Provincial Dairy Analyst. The stock *Bacillus subtilis* culture used was supplied by Dr. H. R. Thornton, Professor of Dairying, University of Alberta.

In all cases the organisms were grown in skim milk made by reconstituting skim milk powder on a 1:10 basis, adjusting its pH to approximately 7.0, and autoclaving it at 15 pounds pressure for 20 minutes. Incubation was at room temperature for periods of approximately two weeks.

Where quantitative measurements of volatile acidities are reported, they have been expressed as the equivalent number of cc. of 0.1 N. acid.

Residues obtained by steam distilling acidified skim milk cultures and evaporating the neutralized distillates to dryness are referred to as "steam distillate residues" throughout this thesis.

PART 1

PRELIMINARY INVESTIGATION TO DETERMINE CHARACTERISTIC PROPERTIES OF THE ODOROUS COMPOUND

INTRODUCTION

In order that some of the chemical properties of the compound responsible for the sweaty feet odor of *Ach. putrefaciens* cultures might be determined, it was necessary to investigate some conditions which influenced the intensity of the odor. By making use of one of the properties characteristic of the odorous compound, a concentration of the compound was effected. It was then possible to use the concentrate containing the odorous material for subsequent investigations.

EXPERIMENTAL

It was observed that the sweaty feet odor was not readily discerned from skim milk cultures of *Ach. putrefaciens* which had been made strongly alkaline, but was very pronounced from acidified cultures. Since this observation indicated that the odorous compound might have acidic properties, this possibility was investigated further.

A skim milk culture of *Ach. putrefaciens* acidified with sulphuric acid was steam distilled, the distillate was titrated with sodium hydroxide, and the solution was evaporated to dryness. When this steam distillate

residue was acidified, the characteristic sweaty feet odor was readily observed, but it was accompanied by an acrid odor suggestive of the volatile fatty acids.

An Ach. putrefaciens culture was adjusted to a reaction of about pH 10.0 and steam distilled. The distillate had a pronounced ammonia-like odor, but the sweaty feet odor was not observed.

A solution of the steam distillate residue obtained by distillation of acidified Ach. putrefaciens cultures was adjusted to pH 3.2 and extracted with ether. When a portion of the ether extract was evaporated to dryness, the sweaty feet odor was very pronounced. The remainder of the extract was shaken with sodium hydroxide and the mixture was evaporated to dryness. The sweaty feet odor was again liberated by acidifying the white crystalline residue obtained in this way.

A solution of steam distillate residue was adjusted to pH 8.8 and extracted with ether. The sweaty feet odor was not detected when the ether extract was evaporated to dryness.

These results confirm the assumption that the odorous compound had acidic properties.

Steam distillate residue was prepared from a skim milk culture of B. subtilis. A strong fatty acid odor was evolved when the residue was acidified. To one person interested in the investigation, this odor

bore a resemblance to the sweaty feet odor. Steam distillate residue was also prepared from sterile skim milk. A pronounced fatty acid odor was observed when this residue was acidified. If the compound responsible for the sweaty feet odor of steam distillate residue prepared from Ach. putrefaciens cultures was present at all in residues from sterile skim milk and B. subtilis cultures, it was present only in very low concentrations.

No precipitate formed when a solution of the steam distillate residue from Ach. putrefaciens cultures was saturated with sodium bisulphite, acidified with hydrochloric acid, and allowed to stand overnight. Benedict's Solution was not reduced when it was boiled with a solution of the steam distillate residue. These results indicated that the odorous compound was probably not an aldehyde or a ketone.

A sodium fusion of a specially prepared steam distillate residue was carried out in order to determine the elements which might be present in the odorous compound. A skim milk culture of Ach. putrefaciens acidified with phosphoric acid was steam distilled, the distillate was titrated with sodium hydroxide, and evaporated to dryness. A portion of the residue was fused with sodium, the fused material dissolved in water, and the solution filtered. The solution gave a negative nitroprusside test for sulphur, a negative prussian blue

test for nitrogen, and a negative silver nitrate test for halogens. A solution prepared from a portion of the residue fused with a mixture of sodium carbonate and potassium nitrate, when tested for phosphorus with ammonium molybdate solution, gave a negative result. Since the elements nitrogen, sulphur, phosphorus, and the halogens were not detected in the residue from which the sweaty feet odor was readily released, it seemed probable that the odorous compound might contain only carbon, hydrogen, and oxygen.

SUMMARY

The conclusion drawn from the experiments reported was that the compound responsible for the sweaty feet odor of *Ach. putrefaciens* cultures had acidic properties, was volatile with steam when in an acid solution, and contained no sulphur, nitrogen, phosphorus, or halogens. These results indicated that the compound is probably a volatile acid closely related to the fatty acids of low molecular weight.

PART 2

SEPARATION AND IDENTIFICATION OF VOLATILE ACIDS

INTRODUCTION

It was observed that, in attempts to concentrate the compound responsible for the sweaty feet odor of *Ach. putrefaciens* cultures, the acrid odor similar to that of the lower fatty acids invariably accompanied the sweaty feet odor. It therefore seemed probable that any procedure which would bring about a separation of the volatile acids would at the same time concentrate the odorous compound. For this reason, an investigation of the methods used for studying fatty acid mixtures was undertaken, and some of these methods were applied to the separation of the acids obtained by distillation of acidified *Ach. putrefaciens* cultures.

REVIEW OF THE LITERATURE

Of the many reported methods for the isolation and identification of volatile fatty acids produced by bacterial decomposition, none seems to have given entirely satisfactory results.

Fractional Solution and Precipitation Methods

Linnemann (1871) found basic lead propionate to be soluble in cold water but insoluble in hot water,

whereas the basic lead salts of formic, acetic, and butyric acids were soluble in both hot and cold water. By making use of this property, he separated propionic acid from a mixture of these acids. Luck (1871) separated mixtures of formic, acetic, propionic, and butyric acids by fractional crystallization of their barium salts from absolute alcohol. Haberland (1899) was unable to effect a satisfactory separation of the volatile acids using this procedure, but he separated a mixture of the acids by first precipitating propionic acid by boiling a solution of the basic lead salts, then extracting the zinc salts of acetic and butyric acids with alcohol leaving zinc formate as an insoluble residue, and finally separating acetic and butyric acids by fractional distillation of their amyl esters or by fractional crystallization of their silver salts.

Holzmann (1898) reported that sodium isovalerate is readily soluble in acetone, whereas the sodium salts of the lower members of the fatty acid series are not. By making use of this property, he separated isovaleric acid from propionic, butyric, and caproic acids. Chapman (1899) used this property of sodium isovalerate as the basis for a method of separating isovaleric and acetic acids quantitatively.

Orla Jensen (1904) worked out a procedure for separating the volatile fatty acids by fractional pre-

cipitation of the silver salts, and for estimating them quantitatively by determinations of the percentages of silver in the salts. Phelps and Palmer (1917-2), in discussing methods of separating the volatile acids based on differences in the solubilities of their silver salts, stated: "The more or less complete solubility of these salts either in the alcohol or in the water renders impossible a quantitative separation of these acids in the manner described in the literature." They described a method for the separation of butyric acid from formic and acetic acids based on the differences in the solubilities of the quinine salts in carbon tetrachloride. Crowell (1918) and Parsons and Sturges (1927) were unable to obtain satisfactory results using this technique, but Woods and Clifton (1938) used it in the separation and identification of acetic and butyric acids. Parsons and Sturges used a partial precipitation of the silver salts combined with steam fractionation to separate isovaleric and acetic acids.

Distillation Methods

Duclaux (1874) developed a method of studying volatile acids which has been widely used, and which gives satisfactory results. This procedure is dealt with in detail in the appendix.

Liebig (1849) described a method of separating volatile acids by distilling a partially neutralized

mixture of acids. The weaker acids are liberated first and may be distilled off. Liebig claimed that at least one pure acid could be obtained by a single distillation using this procedure. Duclaux (1874) found that the efficiency of the separation varied with the concentration of the solution, and was unable to effect a complete separation of the acids by this procedure. Wechsler (1893) reported a method of separating the volatile acids based on the same principle as that used by Liebig. Crossley (1897) repeated Wechsler's experiments, but was not able to obtain a satisfactory separation of mixtures of fatty acids. Holzmann (1898) partially separated acids from acetic to stearic in mixtures by partial liberation of the acids from their alkali salts, followed by distillation of the free acid. Harden (1901), Hart and Willaman (1912), and Currie (1914) used this principle for fractionation of mixtures of the volatile acids in order to simplify subsequent qualitative analysis of the mixtures.

Wolf and Telfer (1917) used a fractionation by steam distillation, but Bushnell (1922) and Wagner, Meyer, and Dozier (1925) found this procedure to be impractical. Olmsted, Whitaker, and Duden (1929) suggested a means of obtaining small quantities of the lowest volatile acid in a mixture in pure condition by the use of steam distillation.

Fractional distillation of either the free fatty acids or their esters has been attempted by numerous investigators, for example, Linnemann (1871), Emmerling (1897), Crossley and Perkin (1898), Dunstan and Henry (1898), Crowther and Hynd (1917), and Hilditch and Jones (1929). This procedure has not generally been found satisfactory for use with the lower volatile acids.

Extraction Methods

Lasserre (1907) stated that acetic and formic acids may be separated from butyric and valeric acids by extracting an aqueous solution of the mixed acids with benzene, in which only the last named acids dissolve, and from which they may be recovered by treatment with baryta solution. Crowell (1918) used a kerosene extraction of the fatty acid mixture to determine quantitatively acetic, propionic, and butyric acids in a mixture.

Osburn, Wood, and Werkman (1936) described a partition method based on the distribution of the fatty acids between two immiscible solvents. By titration of the acids extracted, the partition constants, that is the percentages of the acids which remain in the aqueous phase, may be determined, and the composition of the acid mixture may be calculated from these constants.

A method of separating the fatty acids occurring in cheese depending on the distribution ratio of the acids between butterfat and water at 55°C. has recently

been developed and successfully applied to acid mixtures obtained by extraction of cheese. (Ann. Rep., N.I.R.D., 1939).

Other Methods for the Identification of Acids

Dyer (1916) developed a series of colorimetric tests which can be applied to the purified acids for confirmation of the results obtained by Duclaux distillations or a modification of this procedure. These tests are helpful, but, as pointed out by Wolf and Telfer (1917), they are not specific, and unless the acid is present in sufficient quantity, a positive result will not be obtained.

Phelps and Palmer (1917-2) identified acetic, propionic, and butyric acids by determinations of the melting points of the quinine salts. Drake and Bronitsky (1930) recommended the use of the p-phenylphenacyl esters of organic acids for their identification by melting point determinations.

Musicant and Kaszuba (1939) reported a method for the identification of propionic acid in the presence of acetic and butyric acids by microscopic examination of the mercurous salts of the acids.

EXPERIMENTAL

Preparation of Steam Distillate Residue

Calcium hydroxide was added to a solution of steam distillate residue until precipitation was complete. The

white flaky precipitate which formed was then centrifuged off and washed once with water. The sweaty feet odor was not observed when this precipitate was acidified.

During steam distillation of acidified skim milk or skim milk cultures, small amounts of fatty acids of relatively high molecular weight solidified in the condenser. These higher acids formed insoluble salts of barium and calcium, and could be partially precipitated from the distillates by titration with barium or calcium hydroxide. For this reason, barium hydroxide was generally used for the titration of the distillates.

In order that a uniform supply of a concentrate of the odorous compound might be obtained for the experimental work in this part, a routine procedure was developed for the preparation of steam distillate residue on a rather large scale. Approximately 20 l. of skim milk culture of Ach. putrefaciens were mixed in a 50-litre carboy, the solution was adjusted to below pH 2.0 with sulphuric acid and steam distilled, using steam from the laboratory supply line. Approximately 10 l. of distillate were condensed in a large capacity block tin condenser and neutralized with barium hydroxide. The distillate was then concentrated on a water bath, filtered to remove precipitated material, and evaporated to dryness. The distillation apparatus used for the preparation of the steam distillate residue is shown in Plate 1.

The insoluble material discarded after the concentration of the neutralized distillate was not analysed, but it was assumed that most of the caproic acid and acids above it in the series would be precipitated from the solution by this procedure as the relatively insoluble barium salts.

Oxidation of Formic Acid

It was assumed that formic acid would be present in the steam distillate residue since numerous investigators (e.g., Dakin, Janney, and Wakeman (1913), Evans (1918), Osburn, Wood, and Werkman (1933), and Van Niel (1928)) have reported that formic acid is produced during the distillation of an acidic carbohydrate solution. In some of the experiments reported later, the analysis of the volatile acid mixture was simplified to a large extent by eliminating the formic acid by oxidizing it to carbon dioxide and water with mercuric oxide, following a procedure similar to that described by Osburn, Wood, and Werkman (1933). The efficiency of this procedure was checked on a known mixture of formic and acetic acids. The Duclaux constants for a mixture containing the equivalent of approximately 10 cc. of formic acid and 40 cc. of acetic acid were determined. The acids were then liberated from their salts by the addition of sulphuric acid, refluxed with an excess of mercuric oxide for thirty minutes, distilled to recover the acids, titrated, and again Duclaux distilled. The

complete destruction of the formic acid was indicated by the close agreement of the constants for the acid left after the oxidation with those for pure acetic acid. (Table 2)

Separation of Acids by Fractional Solution and Precipitation Methods

Attempts were made to separate the volatile acids in the steam distillate residue by fractional precipitation of their silver salts, but silver fractionation methods proved to be of little value as long as the odor of the fractions was the only criterion used to follow the progress of the fractionations. Subsequently silver precipitations were found to be very helpful when Duclaux distillations of the acid fractions were used to determine the compositions of the fractions. In an experiment described later a silver fractionation was used along with steam fractionation for the separation of isovaleric acid.

The procedure suggested by Phelps and Palmer (1917-2) based on the fractional solution of the quinine salts of volatile acids in carbon tetrachloride was followed in attempts to fractionate the volatile acids present in the steam distillate residue, but the attempted preparation of the quinine salts was not successful.

The procedure suggested by Chapman (1899) for separating acetic and isovaleric acids was applied to the steam distillate residue. Approximately 5 gm. of steam

distillate residue were converted into the sodium salt and boiled with 250 cc. of acetone containing 0.5% water. The solution was filtered while hot and the acetone evaporated off. The residue dissolved by the acetone weighed less than 0.05 gm. The sweaty feet odor was released from both the acetone soluble and the acetone insoluble salts when they were acidified. Duclaux constants were determined for the acids present in the acetone soluble salt. A comparison of these constants with those for the acids in the original residue (Table 3) indicated that a partial separation of the acids was effected, but the procedure was considered unsatisfactory because of the relatively low solubility of the salts in acetone.

Attempts to identify volatile acids by microscopic examination of crystals of their mercurous salts following the procedure of Musicant and Kaszuba (1939) were unsuccessful.

Benzene Extraction

Extraction with benzene of an aqueous solution of the volatile acids from steam distillate residue was used for the fractionation of the acid mixture. Approximately 20 gm. of steam distillate residue were dissolved in water, the acids were liberated by the addition of sulphuric acid, the precipitated barium sulphate was centrifuged off, and the clear solution was made up to a volume of 250 cc. It

was then shaken with 50 cc. of benzene, the benzene was separated in a separatory funnel, and the extracted acid was removed by shaking the benzene with barium hydroxide in the presence of phenolphthalein. The acid solution was extracted twice more with the same benzene. The amounts of acid recovered by the three extractions were 135.1 cc., 73.8 cc., and 43.5 cc. Duclaux constants were determined for each acid fraction. From an inspection of the constants for the acids in the original residue, the first fraction, and the acids remaining after the extraction (Table 4), it is evident that a definite fractionation of the volatile acids was effected.

Distillation Methods

Approximately 20 gm. of a mixture containing propionic, butyric, and caproic acids were fractionally distilled in order to determine the value of such a procedure applied to the separation of fatty acids of low molecular weight. Even with the relatively large amount of acid mixture used, compared with the amount which would be available if fractional distillation were used for the separation of the fatty acids encountered in this study, the separation was far from complete. It was concluded that fractional distillation would be of little value in this investigation.

Attempts were made to separate known volatile acid

mixtures as well as the unknown mixture from the steam distillate residue by partially liberating the acids from their salts and distilling off the liberated acids. A solution of 0.4 gm. of valeric acid in water was slightly over-neutralized, then 0.4 gm. of butyric acid was added and the solution was Duclaux distilled. The volatile acids remaining in the residue were liberated with sulphuric acid and the solution again Duclaux distilled. A comparison of the constants for these distillations (Table 5) with constants for butyric and valeric acids indicated that a partial separation of the acids was obtained, but the separation was not as complete as Liebig (1849) claimed could be effected.

For the application of this procedure to the fractionation of the volatile acids in the steam distillate residue, a weight of residue which contained the equivalent of 200 cc. of 0.1 N. acid was dissolved in water and the acids were liberated in five fractions by the addition of 4 cc. portions of N. sulphuric acid. Duclaux constants were determined for each of the fractions. The sweaty feet odor was readily observed from the first two fractions, it was questionably detected from the third fraction, and was not detected from the last two fractions. Table 6 contains the Duclaux constants for the first, third, and fifth fractions. The extent to which the acids from the

steam distillate residue were separated by this procedure may be estimated from an inspection of these results.

Steam Fractionation

A steam distillation procedure similar to that used by Parsons and Sturges (1927) for the fractionation of volatile acid mixtures was applied to the steam distillate residue. A solution containing the equivalent of approximately 1000 cc. of 0.1 N. volatile acid released from steam distillate residue was fractionated by steam distillations at a constant volume of 150 cc. following the procedure described by Dyer (1916). The tables determined by Dyer for his modification of the Duclaux method were used as guides to indicate the volumes of distillates which would have to be collected in order that the various volatile acids would be removed completely from the distillation flask. The data for the fractionation are summarized in Table 7.

The progress of the steam fractionation was followed by Duclaux distillations. It was subsequently found that four acids were present in the original mixture. Since it is doubtful whether calculations of four acid mixtures based on single Duclaux distillations may be considered reliable, the compositions of the first fractions were not calculated. Table 8 contains the constants for fractions A, B, and C; the constants

for fractions D and E were only slightly lower than those for fraction C. Later fractions from the steam fractionation contained only three acids, or at least only very small quantities of a fourth acid, so that the compositions of these fractions could be calculated. Table 9 contains the constants for fractions D and B8, along with constants calculated for mixtures of formic, acetic, and butyric acids. It was expected that fraction B_8 would contain a small amount of isovaleric acid along with the other acids, since only 330 cc. of distillate were collected before fraction B_8 in the fifth fractionation, whereas Dyer's tables indicate that it would be necessary to collect more than 400 cc. to remove the isovaleric acid completely. The poor agreement of the last three calculated constants with the experimentally determined constants probably resulted from the presence in the mixture of a small amount of isovaleric acid which was neglected in the calculations. Table 10 contains the constants for fractions B_9 and B_{10}, and also constants calculated for mixtures of acetic, butyric, and isovaleric acids. From an inspection of these tables, it is evident that a satisfactory separation of isovaleric acid and of a mixture of acetic and formic acids was effected.

Calculations of the compositions of the fractions based on Duclaux constants indicated that butyric acid

was present in the steam distillate residue, but a separation of this acid from the other acids in the mixture was not effected by steam fractionation. However, it was considered that the Duclaux constants provided sufficient evidence to justify the conclusion that butyric acid was present, particularly after the presence of acetic and isovaleric acids had been confirmed. When two of the acids in a three-acid mixture are known, the Duclaux method gives a more reliable identification of the third acid.

Identification of Acetic Acid

Fractions C, D, E, B_2, and B_8 from the steam fractionations were mixed and used for experiments to confirm the identification of acetic acid. The formic acid was destroyed by refluxing the solution of the free acids with mercuric oxide, and the acids remaining were recovered by distillation. Duclaux constants for the recovered acid mixture indicated that some higher acids were present along with the acetic acid. Therefore another steam fractionation was used for the purification of the acetic acid. The acid solution was steam distilled at a constant volume of 150 cc. The first 400 cc. of distillate collected were titrated, concentrated, the acids liberated by the addition of sulphuric acid, the clear solution made up to a volume of 150 cc., and again steam distilled. The

first 400 cc. of distillate collected from this distillation were discarded; the distillates collected after the first 400 cc. from both distillations were mixed, titrated, and concentrated. From an inspection of Dyer's tables, it was assumed that each of these fractionations would remove 98.75 per cent. of the isovaleric acid and 97.68 per cent. of the butyric acid present in the mixture distilled. The purified acetic acid was Duclaux distilled; the first 70 cc. of distillate were collected as fraction I and the balance of the acid as fraction II. Each of these fractions was Duclaux distilled. The constants for these fractions (Table 11) furnished proof that the acid was pure acetic acid, not a mixture of acids. The Duclaux constants for two fractions obtained by distillation of an acid solution would not be the same if the solution contained a mixture of acids.

Dyer's colorimetric test for acetic acid, when applied to the acid remaining after the Duclaux distillation of one of the fractions from the steam fractionation, gave a definitely positive reaction. Dyer's tests cannot be considered reliable because they are not specific and they require comparatively high concentrations of acids to give positive results. However, they may be used to supply additional evidence for the confirmation of identifications of volatile acids.

The purified barium acetate prepared for the determination of Duclaux constants and the experimentally produced and purified barium acetate were used for the preparation of quinine acetate for melting point determinations following the procedure used by Phelps and Palmer (1917-2). The salts were purified by recrystallizing them twice from ethyl acetate and dried in a vacuum desiccator over potassium hydroxide and sulphuric acid. The melting points for the experimental product, the authentic quinine acetate, and the two products mixed were determined using three melting point tubes attached to one thermometer. Table 12 contains the uncorrected results for three determinations, along with melting point data for a derivative of isovaleric acid. Since the melting point of quinine acetate is really a decomposition point, it was considered that these data showed satisfactory agreement. Phelps and Palmer reported the melting point of quinine acetate as 124° - 126° C.

Identification of Isovaleric Acid

Fractions B_9 and B_{10} were mixed and used for experiments to confirm the identification of isovaleric acid. The isovaleric acid was fractionally precipitated as the silver salt by the addition of 4 cc. portions of N. silver nitrate. Table 13 contains the Duclaux constants for the acids liberated from the first and last precipitates. These

constants showed that some lower volatile acid was precipitated as the silver salt along with isovaleric acid.

Part of the acid precipitated as the silver salt was distilled following the Duclaux procedure. The first 20 cc. of distillate were collected as fraction I and the next 50 cc. as fraction II, then each fraction was again Duclaux distilled. The last 20 cc. of distillate were discarded since they contained a large part of the acetic acid remaining as an impurity in the isovaleric acid. The constants for the two fractions (Table 14) were not identical, but they showed sufficiently close agreement to be considered as additional proof that the acid was relatively pure isovaleric acid.

Dyer's colorimetric test for valeric acid, when applied to a portion of the acid which had been precipitated as the silver salt, gave a definitely positive result.

Unsuccessful attempts were made to prepare the quinine salt of the purified isovaleric acid following the same procedure as that used for the preparation of quinine acetate. The quinine isovalerate was apparently so unstable that it changed to the quinotoxine salt while in solution. (Phelps and Palmer, 1917-1). This result provides a probable explanation for unsuccessful attempts

to apply the method of Phelps and Palmer (1917-2) to the separation of volatile acids in mixtures containing isovaleric acid. (Page 17 and also Parsons and Sturges, 1927).

The purified isovaleric acid from the Duclaux distillations was converted into the sodium salt and used for the preparation of the p-phenylphenacyl derivative following the procedure described by Drake and Bronitsky (1930). The solid ester was recrystallized twice from 50 per cent. alcohol and dried in a vacuum desiccator. The uncorrected melting points of this product, the derivative prepared by the same procedure from authentic isovaleric acid, and a mixture of the two products were determined and are included in Table 12. Drake and Bronitsky reported the melting point of p-phenylphenacyl isovalerate as 76° (Uncor.) These results confirmed the identification of isovaleric acid.

SUMMARY

Several methods which might be used for the separation and identification of volatile acids distilled from acidified skim milk cultures of *Ach. putrefaciens* were investigated.

Part of the acid present in the steam distillate residue was destroyed by oxidation with mercuric oxide.

Since it was expected that formic acid would be present in the residue, the acid lost by oxidation was assumed to be formic acid. Duclaux distillations also provided evidence that formic acid was present, and that it was destroyed by oxidation with mercuric oxide.

The method developed by Phelps and Palmer based on the fractional solution of the quinine salts of the volatile acids in carbon tetrachloride did not give satisfactory results with the volatile acid mixture from the steam distillate residue. Attempts to prepare quinine isovalerate for the identification of isovaleric acid by the melting point of its quinine salt were also unsuccessful.

It was decided that fractional distillation would not be applicable to the volatile acids encountered in this study because of the large amounts of the acids which would be required to obtain satisfactory results.

A partial separation of the volatile acids was effected by four procedures: (1) Extraction of the sodium salts of the acids with acetone, (2) Partial liberation of the acids followed by distillation, (3) Extraction of an aqueous solution of the acids with benzene, and (4) Steam fractionation. Of these procedures, the last two were considered the most satisfactory.

A satisfactory separation of isovaleric acid and of a mixture of formic and acetic acids was effected by the use of several steam fractionations. The acetic acid was purified by destroying the formic acid with mercuric oxide and by steam fractionation to remove the higher acids. It was identified by Duclaux distillations of the fractionated acid solution, by Dyer's colorimetric test, and by the melting point of its quinine salt. The isovaleric acid was purified by means of a silver fractionation and identified by Duclaux distillations of the fractionated isovaleric acid, by Dyer's colorimetric test, and by the melting point of its p-phenylphenacyl ester.

Duclaux distillations were used to follow the progress of all fractionation procedures.

PART 3

QUANTITATIVE DETERMINATIONS OF VOLATILE ACIDS IN STEAM DISTILLATE RESIDUES

INTRODUCTION

When acidified sterile skim milk was steam distilled, a relatively high yield of residue was obtained from the titrated distillate. Therefore, in order to determine the effect of the growth of Ach. putrefaciens in skim milk on the volatile acids recovered by steam distillation of the acidified milk, it was necessary to investigate the quantitative composition of the residues from both sterile milk and from Ach. putrefaciens cultures. Skim milk cultures of B. subtilis were also investigated so that the results of the distillations of Ach. putrefaciens cultures might be compared with those of a common proteolytic organism.

EXPERIMENTAL

Duclaux distillations were used for the quantitative estimations of the volatile acids on the assumption that the acids present in the mixtures would be the same as were found in the residue used in Part 2. The constants calculated for mixtures of acetic, butyric,

and isovaleric acids showed sufficiently close agreement with experimentally determined constants to justify this assumption.

For the quantitative determinations of volatile acids obtained by steam distillation of skim milk, 1-litre portions of sterile skim milk were acidified to below pH 2.0 by the addition of 50 cc. of 60 per cent. phosphoric acid and steam distilled at constant volume until 4 l. of distillate were collected. The distillates were titrated with barium hydroxide and concentrated. The volatile acids were liberated from the barium salts by the addition of sulphuric acid and again distilled and titrated. This second distillation made possible a more accurate determination of the volatile acidities of the distillates than could be obtained by the titration of the large volumes of distillate. Duclaux constants were determined for the volatile acids from each distillation. Table 15 contains five typical sets of constants and the total volatile acid obtained by each distillation, expressed as cc. of 0.1 N. acid per litre of milk distilled.

The volatile acids from a number of distillations were bulked and the formic acid present was destroyed by oxidation with mercuric oxide. The remaining acids were recovered by distillation and estimated by titration.

The acids were again liberated by the addition of sulphuric acid, the solution was shaken with benzene until sufficient volatile acid for a Duclaux distillation was extracted, then Duclaux constants were determined for both the extracted acid and for that remaining after the extraction. This benzene extraction was included as a check on the qualitative composition of the volatile acid mixture as calculated from the Duclaux constants. If two acids intermediate between acetic and isovaleric acids were present in the mixture instead of butyric acid, it is highly improbable that the two acids would be extracted by benzene in such proportions that calculations based on the constants for the extracted acid and for that remaining after the extraction would both indicate the presence of butyric acid. The constants for the acids extracted with benzene, together with constants calculated for a mixture of acetic, butyric, and isovaleric acids, are presented in Table 18. This table also contains the corresponding data for distillations of cultures of _Ach. putrefaciens_ and _B. subtilis_.

The quantitative composition of the volatile acid mixture from the sterile milk residue is summarized in Table 19, along with corresponding data for cultures of _Ach. putrefaciens_ and _B. subtilis_. The total acid reported is the average for the second distillations of

the residues used, expressed as cc. of 0.1 N. acid per litre of milk distilled. The figure reported for formic acid is the difference between the acidities before and after the oxidation, since the loss of acidity was assumed to be due to the destruction of formic acid. No corrections were made for other losses of acid in the experimental procedure so that this figure can only be considered a rough estimation of the formic acid originally present in the residue. The quantities of acetic, butyric, and isovaleric acids were calculated from analytical data obtained by Duclaux distillations.

This procedure for the analysis of the volatile acids obtained by steam distillation of acidified skim milk was also applied to acids distilled from skim milk cultures of *Ach. putrefaciens* and of *B. subtilis*. The 1-litre portions of the cultures were incubated at room temperature for periods varying from 12 to 22 days. They were steam distilled and the acid distillates were analysed by the same procedure as that used for sterile milk. Typical constants for distillates of *Ach. putrefaciens* cultures are presented in Table 16, and for distillates of *B. subtilis* cultures in Table 17. Other data for these experiments are tabulated along with the corresponding data for the sterile milk analysis in Tables 18 and 19.

SUMMARY

Sterile skim milk, *Ach. putrefaciens* cultures, and *B. subtilis* cultures were acidified with phosphoric acid and steam distilled. The formic acid present in the distillates was destroyed by oxidation and the remaining acids were determined quantitatively by means of Duclaux distillations.

DISCUSSION

In order that valid conclusions may be drawn from the results reported, a discussion of certain difficulties encountered in the investigation and a critical examination of the techniques used are pertinent.

Observations of Odor Intensity

Throughout the investigation, difficulty was encountered in following the odorous compound through various treatments. The only criterion which could be used as a guide for the concentration of the compound was its odor. This criterion is very unsatisfactory for use in experimental work for several reasons.

1. There is no adequate method of describing odors. We have definite units for measuring the color of a solution, the intensity of light, the pitch of a sound, etc., but the description of either the nature or the intensity of odors still presents great difficulty. Odors are generally described by reference to something with a similar odor, or by specifying the compound giving the odor if this is known. However, certain odors are encountered which cannot be adequately described by comparison with familiar odorous materials; then it is only possible to describe such an odor by the use of common, general terms such as disagreeable, pungent,

fruity, sour, etc. The methods used for expressing odor intensities are likewise in a very unsatisfactory state.

2. One odorous compound may so change or mask the odor of another compound that the latter cannot be recognized by smell. This factor became important in this investigation because, as the odorous compound was concentrated and the intensity of the sweaty feet odor increased, it was masked to some extent by an increase in the intensity of the familiar, pungent, fatty acid odors.

3. The environment in which odor observations are made may markedly influence their results. In the course of this study, it was consistently observed that atmospheric conditions had a definite influence on the sweaty feet odor and the ease with which it was detected. The temperature and the humidity seemed to be the most important factors involved, but the concentration of the odorous compound, the rate of circulation of air, and similar variable conditions also caused inconsistencies in the odor intensities. Attempts to standardize the conditions for the odor observations were not successful. The difficulty of deciding whether the odorous compound was present at different stages in experiments was increased by the persistency of the odor where the work was being done, and by the difficulty of keeping the hands free from it.

4. The organs of smell lose their sensitivity under certain conditions, such as when the observer has a "cold", or after inhalations of a compound with a pungent odor or with an anaesthetizing effect. Observations made while the sensitivity of the olfactory organs is not normal may be misleading.

Separation and Identification of Volatile Acids

The separation and identification of volatile fatty acids in mixtures has always been a difficult problem for workers in the biological sciences.

In the present study, the extraction of an aqueous solution of the free fatty acids with benzene proved to be a convenient, rapid method for the preliminary fractionation of a volatile acid mixture. This method gave a much more complete fractionation of the acid mixture encountered and in a shorter time than either steam fractionation or Liebig's method of partial liberation of the acids from their alkali salts, followed by distillation.

The estimation of formic acid by determining the reduction in the volatile acidity resulting from oxidation of the free acids with mercuric oxide gave sufficiently accurate results for the purposes of this investigation. For more accurate quantitative determinations, it would probably be necessary to use a method

such as that described by Shaffer and Friedemann (1924) or by Weihe and Jacobs (1936). After the elimination of formic acid by oxidation, the subsequent analysis of the acid mixture remaining was simplified.

For the isolation of individual volatile acids from the fractionated mixtures, steam and silver fractionations gave the most satisfactory results.

The Duclaux method was very useful for both qualitative and quantitative determinations of the volatile acids; to indicate the efficiency of fractionation procedures, and to identify the fractionated acids. The Dyer steam distillation procedure was used for a number of distillations, but it did not seem to offer sufficient advantage over the Duclaux method to warrant its use. In investigations of volatile acid mixtures, the use of the partition method of Osburn, Wood, and Werkman should be given serious consideration, particularly for routine analyses of a known acid mixture.

Separation of Volatile Acids by Steam Distillation

Steam distillation of acidified solutions has frequently been used for the separation of volatile acids from materials encountered in a variety of investigations (e.g., Dunstan and Henry, 1898; Suzuki, Hastings, and Hart, 1910; Hart and Willaman, 1912; Currie, 1914;

Evans, 1918; Bushnell, 1922; Hammer and Sherwood, 1923; Wagner, Meyer, and Dozier, 1925; Parsons and Sturges, 1927; Van Niel, 1928; and Hillig and Clark, 1938). Recently the validity of conclusions based on estimations of volatile acids obtained in this way has been questioned. It is therefore desirable to discuss critically the steam distillation procedure used in this study.

In the "Manual of Methods for Pure Culture Study of Bacteria" edited by the Committee on Bacteriological Technic of the Society of American Bacteriologists, it is stated that: "Separation of the volatile acids is ordinarily effected by steam distillation after acidification with sulphuric acid to pH 2.0 to liberate the acids. It is necessary to collect twelve volumes of distillate; e.g., 300 ml. from 25 ml. of medium, in order to remove the volatile acids quantitatively." However, in most of the investigations listed above, not more than 4 or 5 volumes of distillate were collected.

Hiscox and Harrison (1938) studied the power of cheese and its constituents to retain volatile acids during steam distillation. They found that in general, the accelerating or retarding action of certain constituents of cheese was of such a nature that the

the relative proportions of the volatile acids in fractions collected during the early stages of distillation bore little relationship to their original distribution in cheese. The discrepancies were most marked for the highest and lowest members in the series of acids studied. The difficulty caused by the retentive power of the fat was evaded by continuing the distillation long enough to overcome its action. Even then, it appeared that one or more of the constituents of the cheese, probably break-down products of the casein, still held an undetermined percentage of some of the acids so strongly that they could not be removed by simple steam distillation. Hiscox, Harrison, and Wolf (1938) found that by water extraction of the cheese and alkaline extraction of the fat, followed by ether extraction of the residues, a value for volatile acids of cheese was obtained which was from 2 to 4.5 times higher than that from direct steam distillation. They therefore questioned the validity of the conclusions frequently reported in the literature that have been drawn from estimations of volatile acids based on direct steam distillation of cheese. Marquardt and Dahlberg (1940) reported results similar to those obtained in the above investigation. The results of these investigations indicate that steam distillation

of acidified solutions containing milk solids cannot be considered as a satisfactory procedure for the quantitative removal of volatile acids.

If the volatile acid values obtained by steam distillation in other studies, such as those of Suzuki, Hastings, and Hart (1910), Bushnell (1922), and Hiscox and Harrison (1938), are calculated on the basis of the weight of milk solids-not-fat in the solutions distilled, these calculated values are somewhat lower than those obtained in this investigation by steam distillation of acidified sterile skim milk. The sources of the initial volatile acidity were not determined. It is possible that the conditions for the steam distillation caused the production of free volatile acids from certain milk constituents. It was considered improbable that bacterial growth in the milk before the spray drying would have caused a significant increase in the volatile acidity.

Reference has already been made to workers who have reported that formic acid is produced during the steam distillation of acidic carbohydrate solutions. Evans (1918) used phosphoric acid in place of sulphuric acid to acidify milk cultures for distillation because, when sulphuric acid was used, formic acid was formed during the distillation. The results reported in Table 19 show that even when phosphoric acid was used, formic acid made up more

than one-third of the acid distilled from sterile milk. Since this value was approximately the same for the cultures of Ach. putrefaciens and B. subtilis also, it seemed probable that the formic acid arose from some constituent of the milk which was not changed to any great extent by the action of the bacteria, e.g., the lactose.

The quantitative determinations of formic acid have little significance because the formic acid was not completely removed by the distillation procedure employed, and at least part of the formic acid was probably produced by the distillation technique. For this reason, little attempt was made to apply corrections for experimental losses in the formic acid estimations. For quantitative determinations of formic acid in urine, Dakin, Janney, and Wakeman (1913) used an ether extraction to remove the formic acid with a minimum of chemical manipulation. It would probably be necessary to apply a similar extraction technique for the estimation of formic acid in milk cultures in order that the results might be considered reliable.

A medium such as milk is not very satisfactory when used in studies of volatile acid production by bacteria because the initial volatile acidity is so high that it makes up the greater part of the total volatile

acidity of the cultures. It cannot be taken for granted that the increase in volatile acidity observed in this study resulted from the production of acids in the free form by the bacteria. It is possible that the increase resulted from a secondary reaction such as the decomposition of partially proteolysed proteins under the combined action of heat and strong acids during the steam distillation.

Several investigators have reported the volatile acid production of different bacteria growing in various media, but they have not reported analyses of the acids distilled from the sterile media nor corrections made for these acids (e.g., Wolf and Telfer, 1917; Wagner, Meyer, and Dozier, 1925; and Parsons and Sturges, 1927). Conclusions which state the volatile acids produced by bacteria, when drawn from such data, cannot be considered valid. In studies of this kind, greater care should be taken to determine and report the volatile acidities of the media as well as of the bacterial cultures.

The Sweaty Feet Odor

Wolochow (1940) found that the odor of skim milk cultures of *Ach. putrefaciens* may be composed of two types of odorous substances differing in chemical behavior. He described the odor components as the "sweaty feet odor" and an "odor typical of putrefaction". When

air which had been passed through *Ach. putrefaciens* cultures was washed with sodium hydroxide, the sweaty feet odor could not be detected, but the odor typical of putrefaction was still observed. The sweaty feet odor was considered the typical and essential surface taint odor. *Ach. putrefaciens* was the only organism which produced the sweaty feet odor in Wolochow's studies, and it was therefore suggested that sweaty feet odor production might be unique to *Ach. putrefaciens*.

Wolochow was not able to detect the sweaty feet odor from skim milk cultures of *Ach. putrefaciens* when the pH was about 7.6 or higher, but observed that the odor was released by acidifying the culture. The sweaty feet odor was not discerned until growth in a litmus milk culture had proceeded beyond the litmus-reduction stage. The E_h of samples of reduced litmus milk cultures of *Ach. putrefaciens* was changed by adding hydrogen peroxide and hydroquinone to the cultures until the litmus was reoxidized to the blue color, and by incubating the cultures at room temperature in an atmosphere of hydrogen gas. In each case, the sweaty feet odor was not observed until the cultures had been exposed to the air for some time. When any of these samples from which the odor had been lost was spread on the fingers and allowed to

dry to the point of stickiness, the sweaty feet odor could be discerned. Wolochow suggested the following possible explanation for these phenomena:

"The 'sweaty-feet' odor material is produced and held in milk solution in a non-odorous reduced form. Exposure to air oxidizes this material to an odorous stage. Furthermore, it would seem that too drastic oxidation will reversibly change the odorous compound to a non-odorous compound, which when it becomes equilibrated with air, again becomes odorous."

One characteristic of surface taint is that it is most pronounced at the surface of affected butter, and that freshly cut surfaces of affected butter do not usually show the defect until they have been exposed to the air for some time. The similarity of this property of the odor of surface taint butter to that of the sweaty feet odor is strong evidence supporting the assumption that the sweaty feet odor is the essential surface taint odor.

From the results of the experimental work reported, it was concluded that the steam distillate residue contained the barium salts of formic, acetic, butyric, and isovaleric acids. In all cases, the sweaty feet odor was concentrated along with isovaleric acid. However a separation of the compound responsible for the sweaty feet

odor from isovaleric acid was not effected. At the same time, the intensity of the sweaty feet odor was not proportional to the concentration of isovaleric acid. For example, from an inspection of Table 19 it may be seen that the steam distillate from B. subtilis cultures contained more than four times as much isovaleric acid as that from Ach. putrefaciens cultures. Although the sweaty feet odor was readily detected from Ach. putrefaciens cultures, only once in all of the experimental work was it detected from B. subtilis cultures. In this case, the sweaty feet odor was observed when the condenser cooling water was not turned on at the start of a steam distillation of an acidified B. subtilis culture and the vapor was allowed to escape. Another observation which showed that the intensity of the sweaty feet odor was not necessarily proportional to the concentration of isovaleric acid was made during the steam fractionation experiment (Table 7). Fractions A_1 and B_3, which had a pronounced sweaty feet odor, and B_6, from which the odor was readily detected, were mixed and fractionated into fractions B_9 and B_{10}. The sweaty feet odor was not detected when these fractions were first examined, but on the following day when the observations were repeated, the odor was very pronounced.

The common odor of isovaleric acid is somewhat similar to the sweaty feet odor, but a person familiar

with the two odors can readily distinguish between them. The sweaty feet odor was not detected from one portion of isovaleric acid purchased for use in this study, but it was readily detected from a less pure "monohydrated" acid purchased from another supply house.

The characteristic odor of perspiration has been attributed to the volatile fatty acids or their esters. Bernthsen (1912), in listing the sources of isovaleric acid, stated: "It is found......in the perspiration from the foot...."

This evidence indicated that there is a definite connection between isovaleric acid and the sweaty feet odor. Under certain conditions of concentration, temperature, humidity, E_h, etc., the common odor of isovaleric acid seems to be replaced by the sweaty feet odor, but further experimental work is necessary to determine the exact relationship between the two odors. However, from the evidence reported, the conclusion that the sweaty feet odor arises either directly or indirectly from isovaleric acid seems justified.

In this study and also in experiments performed by Wolochow (1939-40), attempts were made to produce the surface taint defect in butter by churning cream to which had been added portions of isovaleric acid isolated in various stages of purification from skim milk cultures

of Ach. putrefaciens. Although the odors of some butters produced in this way bore a close resemblance to that of typical surface taint butter, in no case was there complete agreement that such a butter might be considered as typical surface taint. The odor of some of these butters suggested rancidity rather than surface taint. However, since it is to be expected that the odor of surface taint butter would be a mixture of several odors, although one characteristic odor might predominate, it should not be concluded from these experiments that isovaleric acid is not the constituent responsible for the characteristic odor of surface taint butter.

Derby and Hammer (1931) reported that pronounced rancidity sometimes developed in surface taint butter held at comparatively high temperatures. They and also Wolochow (1940) found that Pseudomonas fluorescens produced in butter an objectionable condition which resembled surface taint for two or three days, but this butter soon developed rancidity. Wolochow pointed out that it is sometimes difficult to distinguish incipient rancidity from typical surface taint by smell. Since free volatile acids seem to be at least partially responsible for the characteristic odor of surface taint butter, surface taint could be considered as a type of rancidity.

Source of Isovaleric Acid

It is a commonly accepted fact that volatile acids may be produced by the decomposition of proteins. Bacteria readily bring about reductive deamination of amino acids to give saturated acids, and almost all amino acids are liable to reductive deamination. It has been suggested that although normal volatile acids recovered from bacterial cultures may have a number of sources, any iso acids present usually arise from protein decomposition.

Neuberg and Karczag (1908) reported that on putrefaction, dl-aminoisovaleric acid (dl-valine) underwent deamination and yielded a valeric acid, presumably isovaleric acid. A small part of the amino acid was converted into butylamine. The residual aminoisovaleric acid was laevorotatory in hydrochloric acid so that an asymmetric attack by the bacteria was indicated.

The qualitative composition of the volatile acid mixture isolated in this study was practically the same as that encountered by several other investigators. Wolf and Telfer (1917) found that the growth of _Bacillus sporogenes_ in milk resulted in the production of acetic, butyric, valeric, and caproic acids. Results obtained by Bushnell (1922) indicated that the volatile acid mixture recovered by distillation of milk cultures of a saprophytic

anaerobe contained acetic, butyric, and perhaps valerianic acids. Wagner, Meyer, and Dozier (1925) identified the volatile acids from milk cultures of *Bacillus botulinus* as valeric, butyric, and acetic acids, in roughly the same proportions as they were encountered in this investigation. The acid mixtures isolated from casein digest and beef heart mash cultures of *B. botulinus* had the same qualitative composition. It was concluded that the volatile acids had their origin in protein decomposition products. Parsons and Sturges (1927) identified the acids produced by *Clostridium putrefaciens* in buffered pork medium as acetic, butyric, and isovaleric, the acetic constituting more than three-quarters of the total.

The odor of surface taint butter is suggestive of putrefaction. Derby and Hammer (1931) stated: "Because of the studies already reported and the general nature of the odor and flavor of surface taint butter, it seemed probable that......the general type of decomposition was one involving the protein." They reported that *Ach. putrefaciens* caused proteolysis of milk.

Wolochow (1940) found that the sweaty feet odor was not produced when *Ach. putrefaciens* was grown in raw milk, but was invariably produced in heat-treated milk. Similarly, he was unable to cause the development of surface taint in butter churned from raw cream. It may

be that denaturation of the protein is necessary before Ach. putrefaciens can cause the production of the odorous material. In studying the nitrogen metabolism of Ach. putrefaciens growing in skim milk, Wolochow found that the non-protein nitrogen, the "formol" nitrogen, and the ammonia nitrogen increased with the age of the culture. He suggested that the organism brought about proteolysis of the milk proteins and then reductive deamination of the amino groups in such a way that free fatty acids were produced. The observation that the sweaty feet odor was not detected until the non-protein nitrogen curve began to rise steeply provided attractive evidence that the production of the odorous compound resulted from protein decomposition, but it was cautioned that these might be only unrelated simultaneous occurrences.

The results of this investigation and of the studies reported above favor the conclusion that the isovaleric acid, which is apparently responsible for the sweaty feet odor of skim milk cultures of Ach. putrefaciens, arose from the decomposition of some protein constituent of the milk.

SUMMARY AND CONCLUSIONS

From the results of Duclaux distillations, it was concluded that the acid mixture obtained by steam distillation of acidified skim milk cultures of Ach. putrefaciens contained formic, acetic, butyric, and isovaleric acids. The identification of acetic and isovaleric acids was confirmed by determinations of the melting points of suitable derivatives.

The volatile acids obtained by steam distillation of acidified sterile skim milk, skim milk cultures of Ach. putrefaciens, and skim milk cultures of B. subtilis were determined quantitatively. The formic acid was estimated by titration of the acid mixtures after oxidation with mercuric oxide, and the compositions of the mixtures remaining were calculated from Duclaux constants.

In studies of the volatile acids produced by bacteria, steam distillation of acidified cultures cannot be considered a satisfactory method for the separation of volatile acids from the cultures.

It was concluded that the sweaty feet odor of milk cultures of Ach. putrefaciens arises either directly

or indirectly from isovaleric acid. It also seems highly probable that isovaleric acid is in the same way responsible for the characteristic odor of surface taint butter.

The results reported favor the conclusion that the isovaleric acid was produced by the decomposition of some protein constituent of milk.

APPENDIX

THE DUCLAUX DISTILLATION METHOD

THE DUCLAUX DISTILLATION METHOD

REVIEW OF THE LITERATURE

Application of the Method

The procedure developed by Duclaux (1874) has been used more frequently than any other technique at present available for the study of the volatile acids. Duclaux worked out this method for the determination of volatile acids in wine, but later used it in studies on milk and other materials. Numerous other investigators have used his technique for studying volatile acids, but only a few will be mentioned here.

Richmond (1895) found that the method in the form given by Duclaux did not give satisfactory results, but, by modifying the procedure, Richmond and Miller (1906) were able to estimate the relative concentrations of three acids in a mixture and to determine the acids present.

Suzuki, Hastings, and Hart (1910), Currie (1914), and Evans (1918) used the Duclaux method for volatile acid determinations in studies on the flavor of cheese, and Hart and Willaman (1912) used it in studies on corn silage.

Upson, Plum, and Schott (1917) found that the Duclaux method was not reliable, and claimed that the

theory of the method is not sound. Lamb (1917) found the method reliable, and explained the lack of agreement obtained by Upson, Plum, and Schott as due to impurities in the acids used and to avoidable variations in the experimental conditions.

Whittier and Sherman (1923) obtained very consistent results with the Duclaux method in determinations of the acids produced by bacterial fermentation of lactose. Wagner, Meyer, and Dozier (1925) found that the Duclaux technique gave more satisfactory results than modifications of the method which had been used by other investigators in studies of volatile acids produced by bacteria.

Van Niel (1928), Woods and Clifton (1938), and Clifton (1940) have also used Duclaux's method for studies of the volatile acids produced by pure cultures of bacteria.

Modifications of the Duclaux Method

Richmond (1908) used a smaller volume of solution and collected fewer fractions for titration than required in the original Duclaux procedure.

The Dyer (1916) modification of the Duclaux technique consists of steam distilling the solution of the volatile acids at a constant volume of 150 cc., collecting definite volumes of distillate, and titrating the distillates to determine the percentages of the acid

which distilled in each fraction collected. The distillation rates of the individual acids, when plotted on simple logarithmic charts, appear as straight lines, whereas mixtures of acids give curved lines. The results of the steam distillations may be interpreted by direct comparison of an unknown curve with charts for the individual acids, rather than by the calculations necessary with the Duclaux method. Dyer's procedure has been used by a number of investigators, e.g., Wolf and Telfer (1917), Bushnell (1922), Wagner, Meyer, and Dozier (1924), and Hillig (1939).

Olmsted, Whitaker, and Duden (1929) modified Dyer's technique by saturating the solution of volatile acids with magnesium sulphate. In this way the rates of distillation are considerably accelerated, and the accuracy of the determinations of formic and acetic acids is doubled.

Virtanen and Pulkki (1928) developed a procedure based on the principle of the Duclaux method which requires fewer titrations for the analysis of a mixture of volatile acids.

METHOD

Principle

The Duclaux method of determining volatile fatty

acids is based upon the fact that each of the acids of the series $C_nH_{2n+1}CO_2H$ has a constant rate of vaporization when distilled under standardized conditions. The method consists of distilling 110 cc. of a dilute solution of the free acids, collecting the distillate in 10 cc. fractions, and titrating each fraction. The percentages of acid in each fraction, expressed on the basis of the acidity of 100 cc. of distillate, are generally taken as the Duclaux constants. Since each volatile acid has a definite, characteristic rate of distillation, the Duclaux constants may be determined for each acid, then unknown acids or mixtures of acids may be determined both qualitatively and quantitatively by comparing the Duclaux constants of the unknown with those of the known acids.

The Duclaux Distillation Apparatus

The standardized distillation apparatus shown in Plate 2 was used for all of the determinations in this study. The heat source used was a Cenco Flask Heater with a variable resistance. A 200 cc. round bottom Pyrex flask with a 24/40 standard taper ground glass joint was used as the distillation flask. The flask was connected to a bent glass condenser tube of the specifications shown in the figure. Two 10 cc. volumetric flasks were used as receivers for the distillate. The ground drip

tip on the condenser tube made it possible to fill the receiving flasks to the graduation marks accurately, and facilitated the changing of the flasks without loss of distillate.

The distillation flask and condenser connection were shielded by an insulated metal shield consisting of a can 180 mm. high and 110 mm. in diameter with a slot 25 mm. wide cut along one side to within 10 mm. of the bottom. This was covered with asbestos which was cemented in place by a sodium silicate solution. This shield was easily placed over the distillation flask so that it rested on the heater. It was found that the shield prevented excessive condensation in the distillation flask during distillations, but it did not keep the flask hot enough to cause redistillation of drops which spattered on the walls of the flask.

Procedure

The amount of volatile acid used for the Duclaux distillations was usually approximately equivalent to 40 or 50 cc. of 0.1 N. acid, but some distillations were made with much smaller quantities of the acids. To a solution of the barium salts of the volatile acids, just sufficient N. sulphuric acid was added to completely liberate the volatile acids. When insufficient sulphuric acid was added, the barium sulphate precipitate was not

easily removed, but an excess caused an increase in the rate of distillation of the acids. The precipitate of barium sulphate was removed by centrifugation, was washed once, and the wash water was added to the clear solution of the free volatile acids. The solution was made up to a volume of 100 cc. in a volumetric flask and transferred to the distillation flask. The volumetric flask was rinsed with exactly 10 cc. of water, which was then added to the distillation flask to make the total volume of solution up to 110 cc. From 15 to 25 capillary anti-bump tubes were added, the flask was connected in place in the distillation apparatus, and the electric heater and the condenser water were turned on. The heater was so regulated that approximately 40 minutes were required for the collection of 100 cc. of distillate after the solution had been heated to the boiling point. Two 10 cc. volumetric flasks were used alternately for collecting ten 10 cc. fractions of distillate. One fraction was titrated while the next was being collected. The flasks were not rinsed between fractions but were drained as completely as possible. The fractions were titrated with approximately 0.1 N. barium hydroxide using phenolphthalein as the indicator. The titration readings were taken cumulatively so that it was not necessary to refill the burette to the zero mark after the titration of each fraction.

Calculations

The Duclaux constants used in this study were calculated by dividing the number of cc. of barium hydroxide required for the titration of each fraction by the number required for 100 cc. of distillate and multiplying the result by 100. The constants therefore represent the actual percentages of acid collected in the corresponding volumes of distillate, expressed on the basis of the amount of acid collected in 100 cc. of distillate as 100 per cent.

The algebraic methods described by Gillespie and Walters (1917) were used for the calculation of the composition of acid mixtures containing two or three volatile acids. The compositions of mixtures containing four acids were not calculated from Duclaux constants because it is doubtful whether the results of such calculations can be considered reliable. In a few cases where small quantities of a fourth acid were present in a mixture, the fourth acid was neglected, and calculations were based only on the three acids predominating in the mixture. Constants calculated for these acids did not always show satisfactory agreement with experimentally determined constants, but the extent to which the fourth acid was present could be estimated roughly from the differences between the two sets of constants.

A Monroe Adding-Calculator was used for the numerical computations necessary for the calculations of the results. Three significant figures were carried in all calculations except in the application of the method of least squares to the analysis of three acid mixtures. In this calculation, no figures were dropped until after the necessary subtractions had been completed. The constants for the first and last fractions were omitted from the calculations because it has been observed that there is a disturbing influence at the beginning and end of the distillations (Richmond, 1908).

Several graphical methods for the calculation of the results of Duclaux distillations, including those described by Gillespie and Walters, were studied. It was decided that although graphical methods might be considered satisfactory for rapid, rough calculations of results for the guidance of experimental work, they did not simplify the calculations enough to justify the relatively small loss of accuracy which resulted from their use, especially when a Monroe calculator was available for the algebraic calculation.

Duclaux (1874), Van Niel (1928), Woods and Clifton (1938), and other investigators eliminated the necessity of calculating the composition of unknown

mixtures by comparing the Duclaux constants for the unknown mixtures with constants contained in tables calculated for different known mixtures. The use of this method was considered impractical in a study in which many different unknown mixtures of acids were encountered.

EXPERIMENTAL

Determination of Duclaux Constants

For the determination of the Duclaux constants for the standardized distillation apparatus, the purest acids available were given further treatments for their purification. Glacial acetic acid and propionic acid were converted into their barium salts and crystallized twice from water with ethanol. Butyric acid, valeric acid, and isovaleric acid were silver fractionated in order to separate higher and lower acids, recovered by steam distillation, converted into their barium salts, and finally crystallized twice from water with ethanol. Barium formate was used without further purification. In all cases the salts were dried in a hot air oven at 110° C. and stored in a desiccator over calcium chloride.

Solutions of the barium salts were made up accurately so that they contained the equivalent of between 40 cc. and 50 cc. of 0.1 N. volatile acid in 25 cc.

of solution. Two 25 cc. portions of these solutions were used for the determination of the Duclaux constants, and each portion was distilled twice. The mean values for the four distillations were taken as the Duclaux constants for the apparatus used (Table 1). The percentage of the total acid which was collected in 100 cc. of distillate was also calculated for each acid.

Effects of Variations in the Duclaux Procedure

The effect of using a different distillation flask on the results of the Duclaux distillations was determined by distilling portions of propionic acid from two 200 cc. round bottom flasks. The constants for these two distillations (Table 20) showed that no significant difference resulted from the use of the different distillation flasks.

The effect of varying the amount of volatile acid distilled on the Duclaux constants was studied by distilling the equivalents of approximately 10 cc., 50 cc., and 100 cc. of propionic acid. From the results of these distillations (Table 21) it was concluded that there was no significant difference in the constants obtained by distillations of acids in the different concentrations studied.

In order to determine the effect of the presence of non-volatile compounds in the volatile acid solutions

on the Duclaux constants, propionic acid solutions containing varying concentrations of sodium sulphate and sulphuric acid were distilled. Table 22 contains data for three of these distillations. The results indicated that the presence of non-volatile compounds in the volatile acid solution may cause significant errors in the Duclaux constants.

DISCUSSION

In order that consistent results might be obtained using the Duclaux method, it was found necessary to standardize every step in the procedure.

Duclaux (1874) studied the effect of the size of the distillation flask used on the constants he obtained for acetic acid. He concluded that the size of the flask had little effect on the constants calculated as percentages of the acid in 100 cc. of distillate, but a smaller portion of acid distilled over from the large flask (800 cc. capacity) than from the small flask (300 cc. capacity). Gillespie and Walters (1917) recommended that each investigator prepare his own tables of Duclaux constants from distillations of pure acids made with his own apparatus. The apparatus they used was described in detail, and has been copied and found satisfactory by other investigators, e.g., Parsons and Sturges (1927).

Clark and Hillig (1938) were not able to assemble a steam distillation apparatus which would reproduce the distillation values obtained by Dyer for his modified Duclaux technique. A standardized apparatus was therefore designed which gave consistent results with a number of assemblies. This apparatus was described in detail so that it could be duplicated in other laboratories. Similarly, Hiscox and Harrison (1938) were not able to obtain reproducible results in their steam distillations of volatile acids from cheese until they standardized their apparatus by erecting a battery of distillation units, each consisting entirely of glass, and containing only accurately specified interchangeable parts.

When the present study was begun, it was thought that since the companies which supply laboratory equipment have to some extent standardized their apparatus, it should be possible to assemble a distillation apparatus which could easily be duplicated so that the same Duclaux constants could be used for different assemblies. For this reason, a simple distillation apparatus which can be duplicated more easily than that described by Gillespie and Walters was assembled, and has been described in detail. It is hoped that at a later date it will be

possible to erect other standardized assemblies and determine Duclaux constants for them in order to decide whether distillations from different assemblies show a satisfactory agreement.

Richmond (1895) reported data from which he concluded that the rate of distillation of butyric acid was the same for solutions of concentrations between 0.3 per cent. and 5 per cent., but later he showed that the rates of distillation of formic and acetic acids varied with the concentration of the solutions (Richmond, 1908). Virtanen and Pulkki (1928) found that the concentration of a fatty acid solution had a marked influence on the course of the distillation, even if the solution was very much diluted. In this study, no consistent variations in the constants determined by distillations of equivalents of volatile acid between 10 cc. and 100 cc. were observed. However, the constants calculated from distillations of small amounts of acid were not as accurate as those obtained from larger amounts because of the greater percentage error in reading the burette and in determining the end points of titrations.

Duclaux found that, in general, substances other than volatile acids in the solution to be distilled had little effect on the results of distillations, but he suggested that it would be necessary to eliminate them in

order to obtain accurate results. Virtanen and Pulkki found that neutral salts increased the rates of distillation of acids, but different salts varied in their effects. For example, the rate of distillation of acetic acid was markedly increased in the presence of sodium chloride, but sodium acetate had very little effect. The constants in Table 22 indicated that the presence of sodium sulphate in low concentrations had little effect on the Duclaux constants, but in a higher concentration it caused significant differences in the results. Van Niel (1928) found that an excess of sulphuric acid in a solution used for a Duclaux distillation markedly influenced the results of the distillation. This observation is in agreement with the results of one distillation reported in Table 22. In order to obtain reproducible, accurate results, the volatile acid solutions used for Duclaux distillations in this study were kept as free of foreign materials as possible.

A critical study of the literature on the Duclaux method revealed many differences of opinion regarding the reliability of the procedure. Since there has been so much lack of agreement as to the importance of controlling many different factors influencing the Duclaux distillation, the whole procedure was standardized as much as

possible. In this investigation the method has been found satisfactory for both qualitative and quantitative determinations of volatile acids in mixtures of not more than three acids. Its reliability is greatly increased when it is used along with other methods for the separation and identification of the individual acids in a mixture.

SUMMARY AND CONCLUSION

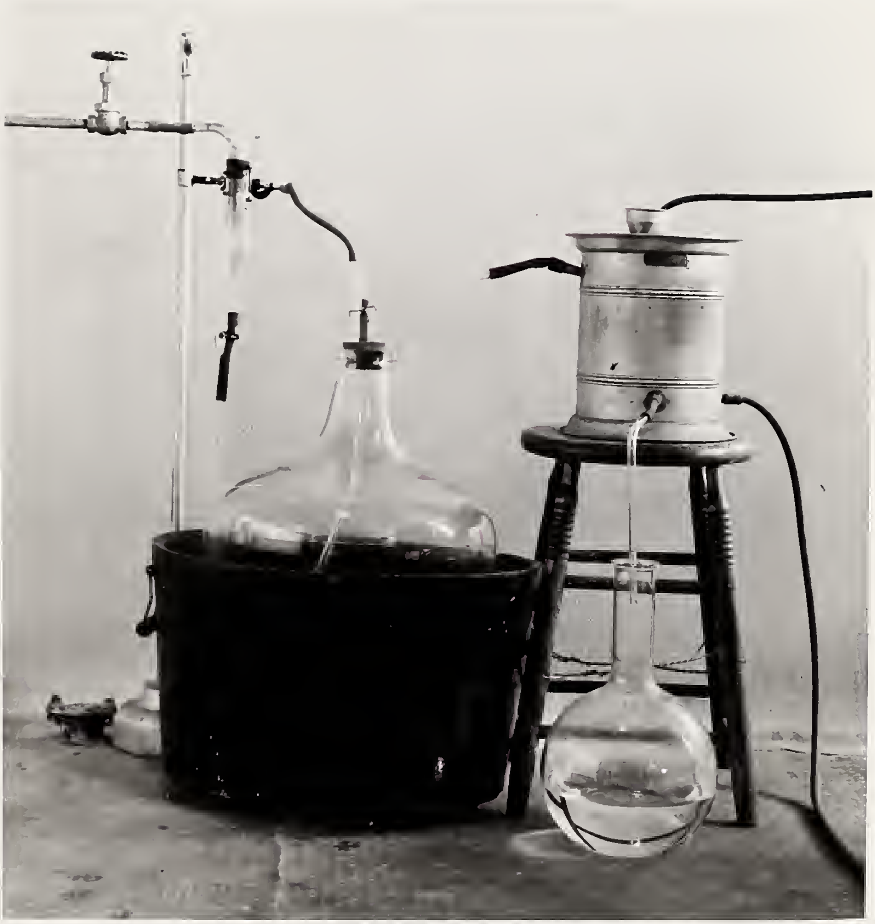
The Duclaux distillation method gives reliable determinations of the qualitative and quantitative composition of volatile acid mixtures if sufficient care is taken to standardize the procedure used.

PLATES

TABLES

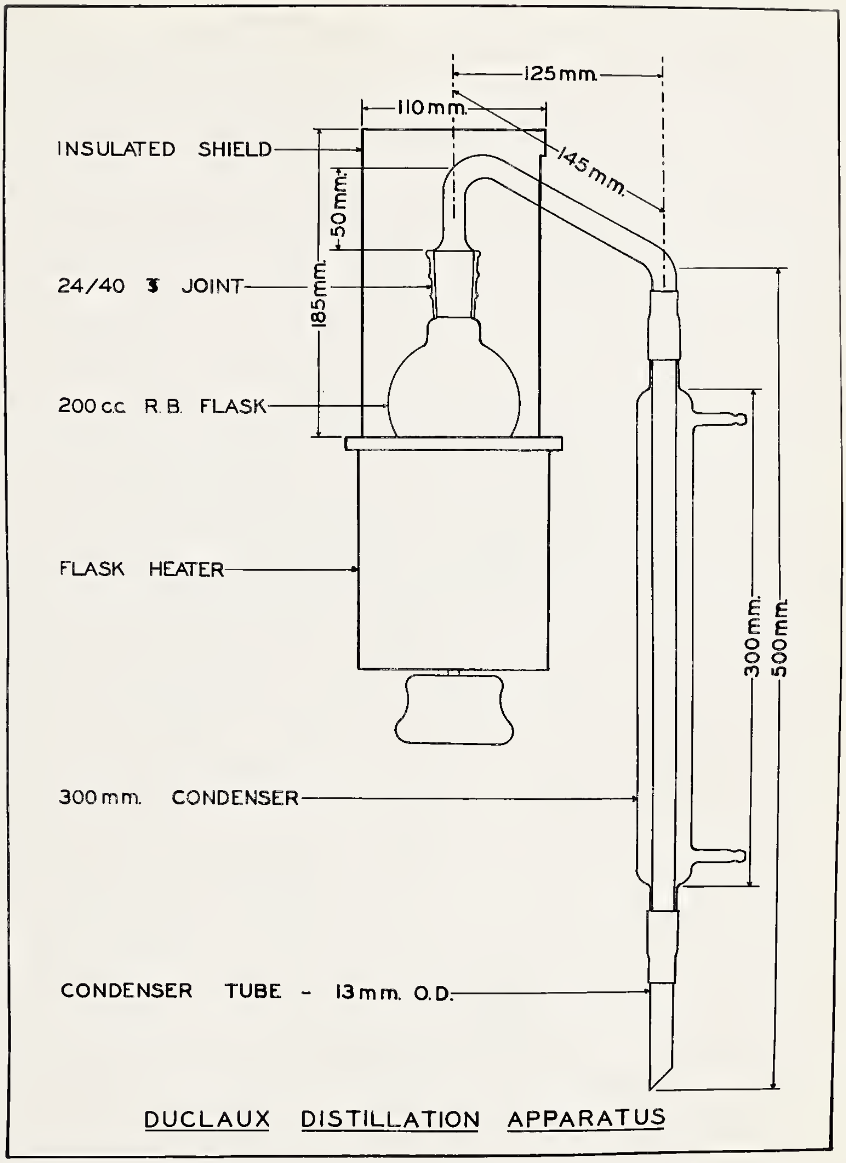
REFERENCES

PLATE 1



STEAM DISTILLATION APPARATUS

PLATE 2



DUCLAUX DISTILLATION APPARATUS

TABLE 1

DUCLAUX CONSTANTS

Volume of Distillate cc.	Percent of Acid in Distillate					
	Formic	Acetic	Pro-pionic	Butyric	Valeric	Iso-valeric
10	5.8	7.7	12.0	17.7	24.8	30.4
20	12.1	15.7	23.6	33.4	44.5	52.9
30	19.0	24.0	34.8	47.4	60.5	69.4
40	26.4	32.5	45.8	59.6	73.1	81.1
50	34.5	41.7	56.2	70.4	82.8	89.2
60	43.4	51.2	66.3	79.5	89.8	94.2
70	53.5	61.5	75.8	87.0	94.6	97.3
80	65.2	72.5	84.7	92.9	97.6	98.8
90	79.8	84.9	92.9	97.3	99.3	99.6
100	100.0	100.0	100.0	100.0	100.0	100.0
Percent of Total which Distilled	62.7	80.1	94.0	98.2	100.0	100.0

TABLE 2

CONSTANTS FOR ACIDS REMAINING AFTER OXIDATION OF FORMIC ACID

Volume of Distillate cc.	Residual Acid	Acetic Acid
10	7.5	7.7
20	15.7	15.7
30	23.9	24.0
40	32.5	32.5
50	41.6	41.7
60	51.3	51.2
70	61.5	61.5
80	72.6	72.5
90	85.0	84.9
100	100.0	100.0

TABLE 3

CONSTANTS FOR ACIDS FROM STEAM DISTILLATE RESIDUE EXTRACTED AS THEIR SODIUM SALTS BY ACETONE

Volume of Distillate cc.	Steam Distillate Residue	Extracted Acids
10	11.8	20.6
20	22.1	36.6
30	31.8	49.0
40	41.0	59.8
50	49.7	68.1
60	58.3	75.6
70	67.5	81.7
80	76.8	87.1
90	87.4	93.2
100	100.0	100.0

TABLE 4

CONSTANTS FOR ACIDS EXTRACTED BY BENZENE FROM A SOLUTION OF THE ACIDS LIBERATED FROM STEAM DISTILLATE RESIDUE

Volume of Distillate cc.	Steam Distillate Residue	First Extract	Remaining Acids
10	11.8	27.2	8.2
20	22.1	47.1	16.5
30	31.8	62.0	25.0
40	41.0	73.1	33.6
50	49.7	81.3	42.6
60	58.3	87.2	52.0
70	67.5	91.5	61.8
80	76.8	94.7	72.5
90	87.4	97.3	84.7
100	100.0	100.0	100.0

TABLE 5

CONSTANTS FOR MIXTURE OF BUTYRIC AND VALERIC ACIDS FRACTIONATED BY METHOD OF LIEBIG (1849)

Volume of Distillate cc.	First Distillation	Second Distillation
10	21.2	19.9
20	38.8	36.6
30	53.7	51.1
40	66.2	63.4
50	76.5	73.5
60	84.7	81.9
70	91.0	88.5
80	95.5	93.7
90	98.4	97.4
100	100.0	100.0

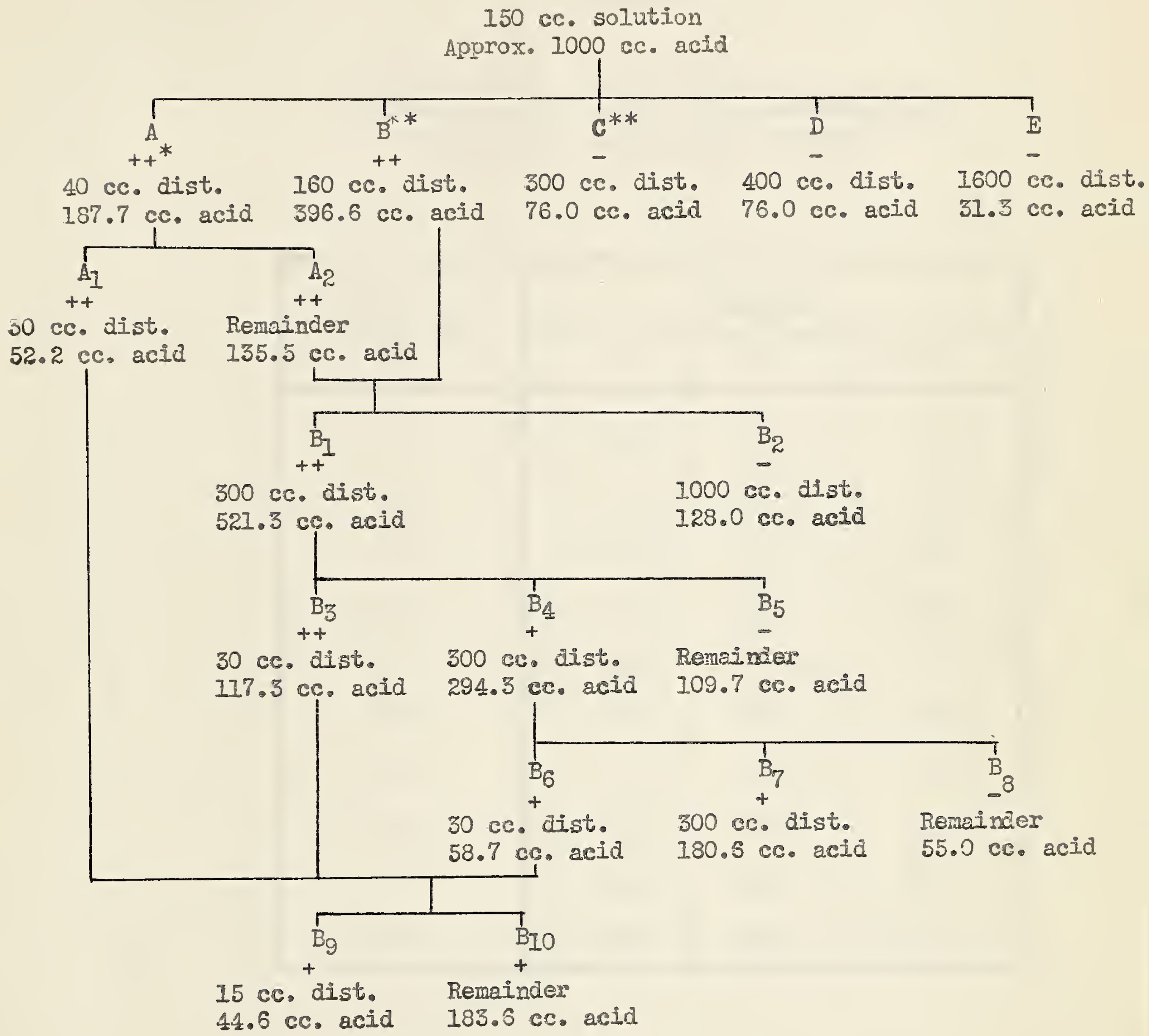
TABLE 6

CONSTANTS FOR ACIDS FROM STEAM DISTILLATE RESIDUE FRACTIONATED BY METHOD OF LIEBIG (1849)

Volume of Distillate cc.	First Fraction	Third Fraction	Fifth Fraction
10	13.4	8.1	6.8
20	25.6	16.6	13.9
30	37.0	25.1	21.6
40	47.7	34.0	29.6
50	57.9	43.0	38.0
60	67.4	52.7	47.2
70	76.5	62.8	57.4
80	84.7	73.8	69.1
90	92.6	85.7	82.7
100	100.0	100.0	100.0

TABLE 7

DATA FROM STEAM FRACTIONATION OF ACIDS FROM STEAM DISTILLATE RESIDUE



* Signs indicate the results of observations for the sweaty feet odor. (++Odor pronounced, + Odor present, – Odor absent). The first figure below the sign gives the volume of distillate collected, the second indicates the equivalent volume of 0.1 N. acid in the distillate.

** Small amounts of acid fractionated in preliminary experiments were added to fractions B and C.

TABLE 8

CONSTANTS FOR STEAM FRACTIONATED ACIDS FROM STEAM DISTILLATE RESIDUE

Volume of Distillate cc.	Fraction		
	A	B	C
10	18.0	11.9	7.6
20	32.4	22.7	15.6
30	44.4	32.4	23.7
40	54.4	41.5	32.4
50	63.0	50.4	41.3
60	70.6	59.2	50.7
70	77.7	67.9	60.8
80	84.5	77.1	71.9
90	91.6	87.3	84.4
100	100.0	100.0	100.0

TABLE 9

CONSTANTS FOR FRACTIONS CONTAINING FORMIC, ACETIC, AND BUTYRIC ACIDS

Volume of Distillate cc.	Fraction D		Fraction B_8	
	Exp.	Cal.[1]	Exp.	Cal.[2]
10	7.1	7.1	7.4	7.4
20	14.5	14.5	15.0	15.0
30	22.3	22.3	23.2	23.0
40	30.5	30.5	31.4	31.3
50	39.2	39.3	40.2	40.2
60	48.5	48.6	49.3	49.5
70	58.7	58.8	59.3	59.6
80	70.1	70.0	70.1	70.7
90	83.1	83.2	82.1	83.6
100	100.0	100.0	100.0	100.0

1. Calculated for 34.5% formic, 65.3% acetic, and 0.2% butyric acids.

2. Calculated for 32.5% formic, 64.6% acetic, and 2.9% butyric acids.

TABLE 10

CONSTANTS FOR FRACTIONS CONTAINING ACETIC, BUTYRIC, AND ISOVALERIC ACIDS

Volume of Distillate cc.	Fraction B_9		Fraction B_{10}	
	Exp.	Cal.[1]	Exp.	Cal.[2]
10	29.1	28.4	22.2	22.0
20	49.8	49.7	39.2	39.2
30	65.5	65.5	52.9	52.9
40	77.0	77.0	63.6	63.6
50	85.2	85.3	72.2	72.3
60	90.7	90.8	79.1	79.1
70	94.5	94.5	84.8	84.9
80	97.0	96.8	90.0	90.0
90	98.6	98.5	94.8	94.7
100	100.0	100.0	100.0	100.0

1. Calculated for 6.6% acetic, 4.0% butyric, and 89.4% isovaleric acids.

2. Calculated for 31.9% acetic, 9.4% butyric, and 58.7% isovaleric acids.

TABLE 11

CONSTANTS FOR FRACTIONATED ACETIC ACID

Volume of Distillate cc.	Fraction	
	I	II
10	7.6	7.6
20	15.7	15.7
30	23.9	24.0
40	32.6	32.6
50	41.7	41.7
60	51.2	51.3
70	61.4	61.5
80	72.5	72.6
90	85.0	85.1
100	100.0	100.0

TABLE 12

MELTING POINT DATA

Experimental Quinine Acetate (a)	126.0°	126.0°	126.0°
Authentic Quinine Acetate (b)	125.5°	125.0°	125.0°
Mixed (a) and (b)	126.0°	126.0°	126.0°
Experimental p-Phenylphenacyl Isovalerate (c)	76.0°	75.5°	
Authentic p-Phenylphenacyl Isovalerate (d)	75.8°	75.8°	
Mixed (c) and (d)	75.8°	75.8°	

TABLE 13

CONSTANTS FOR ACIDS PRECIPITATED AS SILVER SALTS FROM ISOVALERIC ACID FRACTION

Volume of Distillate cc.	First Precipitate	Last Precipitate
10	30.4	29.4
20	52.1	51.2
30	68.3	67.5
40	79.8	79.1
50	87.9	87.1
60	93.1	92.6
70	96.5	96.0
80	98.4	98.1
90	99.5	99.4
100	100.0	100.0

TABLE 14

CONSTANTS FOR FRACTIONATED ISOVALERIC ACID

Volume of Distillate cc.	Fraction	
	I	II
10	30.9	30.3
20	53.0	52.2
30	69.6	69.4
40	81.2	81.1
50	89.2	89.2
60	94.3	94.2
70	97.5	97.3
80	99.0	98.8
90	99.7	99.6
100	100.0	100.0

TABLE 15

TYPICAL CONSTANTS FOR ACIDS OBTAINED BY STEAM DISTILLATIONS OF STERILE SKIM MILK

Volume of Distillate cc.	Flask Number				
	1	2	3	4	5
10	7.9	8.4	8.5	7.9	8.5
20	15.4	16.8	17.0	16.3	16.5
30	23.3	24.7	25.1	23.7	25.5
40	31.5	33.1	33.3	31.6	33.0
50	40.0	42.0	41.8	40.2	41.5
60	49.3	50.9	50.8	49.3	50.5
70	59.3	60.7	60.5	59.3	60.0
80	70.6	71.4	71.4	70.3	71.0
90	83.7	84.2	83.9	83.3	83.5
100	100.0	100.0	100.0	100.0	100.0
Total Acidity	43.1	47.8	41.8	45.3	44.8

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TABLE 16

TYPICAL CONSTANTS FOR ACIDS OBTAINED BY STEAM DISTILLATIONS OF SKIM MILK CULTURES OF *ACH. PUTREFACIENS*

Volume of Distillate cc.	Flask Number				
	1	2	3	4	5
10	8.6	8.7	8.7	8.8	8.8
20	17.0	17.4	17.4	17.3	17.3
30	25.5	26.0	26.2	26.1	26.0
40	34.1	34.7	35.1	34.9	34.8
50	42.9	43.6	44.1	43.9	43.8
60	52.0	52.8	53.3	53.1	53.1
70	61.8	62.7	63.1	63.1	62.9
80	72.2	73.2	73.8	73.6	73.7
90	84.5	85.1	85.7	85.5	85.6
100	100.0	100.0	100.0	100.0	100.0
Total Acidity	65.8	65.3	65.8	69.3	69.8

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TABLE 17

TYPICAL CONSTANTS FOR ACIDS OBTAINED BY STEAM DISTILLATIONS OF SKIM MILK CULTURES OF *B. SUBTILIS*

Volume of Distillate cc.	Flask Number				
	1	2	3	4	5
10	10.0	11.3	12.5	13.2	13.4
20	19.4	21.6	23.6	24.8	25.1
30	28.6	31.1	33.7	35.0	35.7
40	37.5	40.3	43.2	44.5	45.3
50	46.3	49.1	52.3	53.5	54.3
60	55.4	57.9	60.7	62.0	62.8
70	64.7	66.9	69.4	70.3	71.1
80	74.6	76.4	78.5	79.0	79.7
90	86.0	87.1	88.2	88.5	89.0
100	100.0	100.0	100.0	100.0	100.0
Total Acidity	58.9	66.4	73.6	77.9	71.5

TABLE 18

CONSTANTS FOR ACIDS EXTRACTED BY BENZENE FROM MIXTURES REMAINING AFTER THE OXIDATION OF FORMIC ACID

Volume of Distillate	Steam Distillate Residue from					
	Sterile Milk		*Achromobacter putrefaciens* Cultures		*Bacillus subtilis* Cultures	
cc.	Exp.	Cal.[1]	Exp.	Cal.[2]	Exp.	Cal.[3]
10	15.1	15.0	20.6	20.1	26.4	26.5
20	28.1	27.9	36.3	36.2	46.6	46.7
30	39.2	39.1	49.5	49.4	62.2	62.2
40	48.7	48.9	60.0	60.1	73.9	73.8
50	58.1	58.0	68.9	69.1	82.5	82.5
60	66.2	66.3	76.3	76.5	88.6	88.6
70	74.3	74.3	83.0	82.9	92.9	93.0
80	82.4	82.1	88.8	88.6	95.9	96.0
90	90.5	90.4	94.1	94.1	98.2	98.2
100	100.0	100.0	100.0	100.0	100.0	100.0

1. Calculated for 60.4% acetic, 13.2% butyric, and 26.4% isovaleric acids.

2. Calculated for 34.5% acetic, 19.7% butyric, and 45.8% isovaleric acids.

3. Calculated for 6.4% acetic, 19.4% butyric, and 74.2% isovaleric acids.

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TABLE 19

VOLATILE ACIDS FROM STEAM DISTILLATIONS

	Sterile Milk	*Achromobacter putrefaciens* Cultures	*Bacillus subtilis* Cultures
Total Acidity	44.0	65.8	67.4
Formic Acid	17.9	20.1	16.3
Acetic Acid	24.2	40.0	39.5
Butyric Acid	0.2	1.3	2.3
Isovaleric Acid	0.4	1.9	8.1

TABLE 20

CONSTANTS FOR PROPIONIC ACID DISTILLED FROM DIFFERENT DISTILLATION FLASKS

Volume of Distillate cc.	Flask 1	Flask 2
10	11.9	11.9
20	23.4	23.5
30	34.7	34.7
40	45.7	45.8
50	56.2	56.3
60	66.3	66.3
70	75.7	75.8
80	84.6	84.7
90	92.8	92.8
100	100.0	100.0

TABLE 21

CONSTANTS FOR DIFFERENT CONCENTRATIONS OF PROPIONIC ACID

Volume of Distillate cc.	Approximate Equivalent of 0.1 N. Propionic Acid		
	10 cc.	50 cc.	100 cc.
10	11.9	11.9	11.9
20	23.6	23.5	23.5
30	34.5	34.7	34.7
40	45.5	45.8	45.7
50	56.4	56.3	56.1
60	66.2	66.3	66.0
70	75.7	75.8	75.6
80	84.5	84.7	84.5
90	92.8	92.8	92.8
100	100.0	100.0	100.0

TABLE 22

CONSTANTS FOR PROPIONIC ACID CONTAINING COMPOUNDS OTHER THAN VOLATILE ACIDS

Volume of Distillate cc.	Volume of N. Solution of Compound Added			
	0	5 cc. H_2SO_4	5 cc. Na_2SO_4	10 cc. Na_2SO_4
10	11.9	12.1	11.8	12.2
20	23.5	23.8	23.5	23.9
30	34.8	35.2	34.8	35.3
40	45.7	46.1	45.7	46.2
50	56.2	56.6	56.3	57.0
60	66.3	66.5	66.4	67.1
70	75.8	76.2	75.8	76.7
80	84.6	84.9	84.7	85.5
90	92.8	93.0	92.8	93.6
100	100.0	100.0	100.0	100.0

[illegible]

[illegible]

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

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B29747